Diagnostic molecular markers to detect and identify primary parasitoids (Hymenoptera: Braconidae) of *Ericaphis fimbriata* on highbush blueberry

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ABSTRACT

The objective of this research was to develop diagnostic molecular markers for detecting and identifying the most common primary parasitoids of *Ericaphis fimbriata* (Richards) (Hemiptera: Aphididae), which is an important vector of *Blueberry scorch virus* (BlScV) on highbush blueberry *Vaccinium corymbosum* L. (Ericales: Ericaceae) in southwestern British Columbia. Mitochondrial cytochrome c oxidase subunit I gene (COI) sequences and specific reverse primers for the parasitoids *Aphidius ericaphidis* Pike and Starý, *Ephedrus incompletus* Provencher, and *Praon unicum* Smith (Hymenoptera: Braconidae) were developed. The combination of three primers in a multiplex polymerase chain reaction (PCR) assay detected and differentiated DNA from adults of all three parasitoid species. When individual field-collected aphids were challenged with the multiplex PCR assay, immatures of the two numerically dominant parasitoid species, *A. ericaphidis* and *P. unicum*, were readily detected, as was multiparasitism by these two species. The uncommon parasitoid species, *E. incompletus*, was detected less frequently by multiplex PCR assay than by rearing from the aphid hosts.

The diagnostic molecular markers are useful tools for estimating of rates of parasitism and for identifying immatures of parasitoid species within aphid hosts, particularly if used in combination with rearing and dissection assays of field-collected aphids.

Key Words: aphids; parasitoids; mtDNA; COI; multiplex PCR; multiparasitism

INTRODUCTION

Hymenopteran parasitoids attack the dominant colonizing aphid, *Ericaphis fimbriata* (Richards) (Hemiptera: Aphididae), on highbush blueberry, *Vaccinium corymbosum* L. (Ericales: Ericaceae) in the Pacific Northwest (Raworth *et al.* 2008). The two primary parasitoid species most frequently reared from *E. fimbriata* on highbush blueberry in British Columbia (B.C.) are *Praon unicum* Smith and a recently described species previously known as *Aphidius* n. sp. (Raworth *et al.* 2008), now identified as *Aphidius ericaphidis* Pike and Starý (Pike *et al.* 2011). Other primary parasitoids in the genera *Aphidius*, *Praon*, and *Ephedrus* were less frequently reared from *E. fimbriata* in B.C. (Raworth *et al.* 2008); those in the latter genus have since been identified as *E. incompletus* Provencher (Mathur and Pike, unpublished data). All these parasitoids are in the family Braconidae.

Ericaphis fimbriata transmits *Blueberry scorch virus* (BlScV), which causes substantial crop loss (Bristow *et al.* 2000). The primary parasitoids by themselves do not significantly reduce *E. fimbriata* populations on highbush blueberry, but they are part of a broader community of predators and pathogens (Mathur *et al.* unpublished data), which, if conserved, might slow the transmission of BlScV by reducing populations of its main vector. Future studies of the role and impact of primary parasitoids within the community of natural enemies would be facilitated by diagnostic molecular markers that detect parasitoid DNA within

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aphids collected from the field. As noted by Gariepy *et al.* (2007), molecular methods supplement but do not replace traditional rearing and dissection techniques for detecting parasitoids in hosts.

Here we report the development of diagnostic molecular markers that can be used to detect and identify the most common primary parasitoids of *E. fimbriata* on highbush blueberry. Sequence diversity in the mitochondrial cytochrome c oxidase subunit I gene (COI; Hebert *et al.* 2003) was used to develop specific primers for *A. ericaphidis*, *E. incompletus*, and *P. unicum*. Primers for each species were combined in a specific multiplex PCR assay capable of detecting simultaneously the three species of parasitoids. The accuracy of the multiplex assay was validated by sampling a local population of *E. fimbriata* on highbush blueberry and comparing the percentage of collected aphids from which primary parasitoids emerged with the percentage of parasitized aphids detected by PCR.

MATERIALS AND METHODS

Development of specific primers for singleplex and multiplex PCR assays

Adult *A. ericaphidis*, *E. incompletus*, and *P. unicum* that were preserved in 95% ethanol were obtained during field surveys in the Pacific Northwest during 2005 and 2006 (Raworth *et al.* 2008). Genomic DNA was isolated from individual parasitoids with a DNeasy tissue kit® (QIAGEN Inc., Mississauga, ON) using the manufacturer's protocol. Individuals were air-dried to remove ethanol and then homogenized using a disposable microtube pestle (Mandel Scientific Company Inc., Guelph, ON) in the extraction buffer provided with the kit. DNA was eluted in 50 µl of buffer and stored at –80 °C for further use.

The COI region of mitochondrial DNA from individuals was amplified by PCR using the universal insect primer set, LCO 1490 (5'GGTCAACAAATCATAAAGATATTGG) and HCO 2198 (5'TAAACTTCAGGGTGACCAAAAAATCA) (Folmer *et al.* 1994). The PCR mixture (50 µl) contained 1x PCR buffer solution (GeneSys Ltd., Medicorp Inc., Montreal, QC), 0.2 mM of dNTPs, 0.2 µM of each universal primer, 1.25 U of Taq DNA polymerase (GeneSys Ltd., Medicorp Inc., Montreal, QC) and 30–50 ng of DNA template. DNA amplification was performed using a thermal cycler (iCycler, Bio-Rad Laboratories, Mississauga, ON). The temperature regime for all PCR reactions was 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 50°C for 45 s, 72°C for 60 s, and a final extension step of 72°C for 7 min. PCR products were visualized on 1.5% agarose gels stained with GelRed (Biotium Inc., Hayward, CA, USA) and purified with the QIAquick® PCR purification kit (Qiagen Inc., Mississauga, ON). Sequencing of the COI regions was done by the Nucleic Acid and Protein Synthesis Unit (NAPS) at the University of British Columbia (Vancouver, B.C.) using an Applied Biosystem sequencer and the universal primers described above. Polymerase chain reaction products from 13–37 individuals of each parasitoid species were sequenced to check for sequence variability. All sequences were confirmed by sequencing in both directions.

Specific reverse primers were designed for *A. ericaphidis*, *E. incompletus* and *P. unicum* from the regions of the COI gene that were conserved within species but variable among species (Table 1). These primer sequences were evaluated for suitable base composition,

Table 1

Primers used in singleplex and multiplex PCR assays

Primer	Sequence
Reverse *A. ericaphidis*	5' GTCATTACCAATTAACCTACCAGA 3'
Reverse *E. incompletus*	5' GGAAAAGCTATATCTGAACCACC 3'
Reverse *P. unicum*	5' CAGAAATTCCTCTATGTCCAGAA 3'

annealing temperature, and self-compatibility using the on-line software, Primer3 (Rozen and Skaletsky 2000). Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). These primers were evaluated for PCR specificity using DNA from 13–37 individuals from *A. ericaphidis*, *E. incompletus* and *P. unicum*. Polymerase chain reaction conditions and visualization of PCR product were as described previously.

To develop the multiplex PCR assay, the reverse primers for the parasitoids (Table 1) were mixed with universal forward primer LCO in a single reaction tube, and then parasitoid DNA from eight individuals of each of the three species was challenged with the combined primers. Polymerase chain reaction conditions and visualization of the PCR product were as described previously, except that the annealing temperature used in the multiplex assay was 54°C. Extracts of DNA from healthy, unparasitized *E. fimbriata* were included in the assay to check for cross-reactivity.

Validation of the multiplex PCR assay

In 2011, *E. fimbriata* (green and red morphs, alatae and aperterae, immatures except first instars) were collected weekly during May through early September from a 0.15-ha research trial of 6-year-old highbush blueberry 'Duke' plants at the Pacific Agri-Food Research Centre in Agassiz, B.C. (study site reported by Ehret *et al.* 2012). Mature and immature aphids were collected by detaching the leaves on which they were feeding. All aphids were later transferred with a fine paintbrush to the blueberry terminals in buckets described below. The aphid population varied according to trends described by Raworth (2004), therefore the number of aphids collected each week ranged from 65 to 500. About half of the weekly total number of collected aphids were reared to allow parasitoids to develop and emerge (except for the first week, when all collected aphids were reared). The numbers of aphids in rearing were 62, 173, 50, 146, 174, 160, 174, 155, 250, 220, 225, 225, 232, 215, 225, 259, 157, 95 and 51, respectively, for each of the 19 weeks. In the rearing assay, aphids were placed on blueberry terminals cut from plants in the field then washed to ensure they were insect-free. The blueberry terminals stood in a small volume of water in 1.9-litre buckets with mesh lids under natural light at 21 ± 2°C for 30 d. Each week, aphids to be reared were placed in one bucket. The age structure of the sample of aphids was not estimated. Parasitoids that emerged from the group of reared aphids were identified to species (as per Pike *et al.* 2011) and preserved in 70% ethanol. Voucher specimens are stored by K. Pike. Aphids not used in the rearing assay were preserved in 95% ethanol. A subset of these preserved aphids was used for DNA analysis. The multiplex PCR assay was validated by comparing the percentage of aphids that hosted primary parasitoids in the rearing assay with the percentage of preserved aphids that revealed parasitoid DNA. Statistical support for this comparison was generated by calculating the chi-square statistic (Yates' corrected where appropriate; SYSTAT 2007) on the numbers of primary parasitoids that emerged from groups of reared aphids versus the numbers of preserved aphids in which parasitoid DNA was detected by PCR.

RESULTS AND DISCUSSION

Development of specific primers for singleplex and multiplex PCR assays

The extracted mitochondrial DNA from all parasitoid specimens amplified successfully with the universal primers and produced distinct bands on agarose gel. The COI sequences (649 bp) showed high A–T content with an average of 74% of either A or T. The consensus COI sequences for *A. ericaphidis, E. incompletus*, and *P. unicum* (and for *A. ervi* Haliday, *A. matricariae* Haliday, *P. gallicum* Starý, *P. humulaphidis* Ashmead, and *P. occidentale* Baker from an unpublished study by Mathur *et al.*) were deposited in the National Center for Biotechnology Information GenBank under accession numbers EU574902-EU574906, GU237129-GU237131 and KC211020-211032.

All sequences in the sample population of 33 *A. ericaphidis* were identical; therefore, there was only one haplotype (KC211024) and no intraspecific divergence. Within the sample

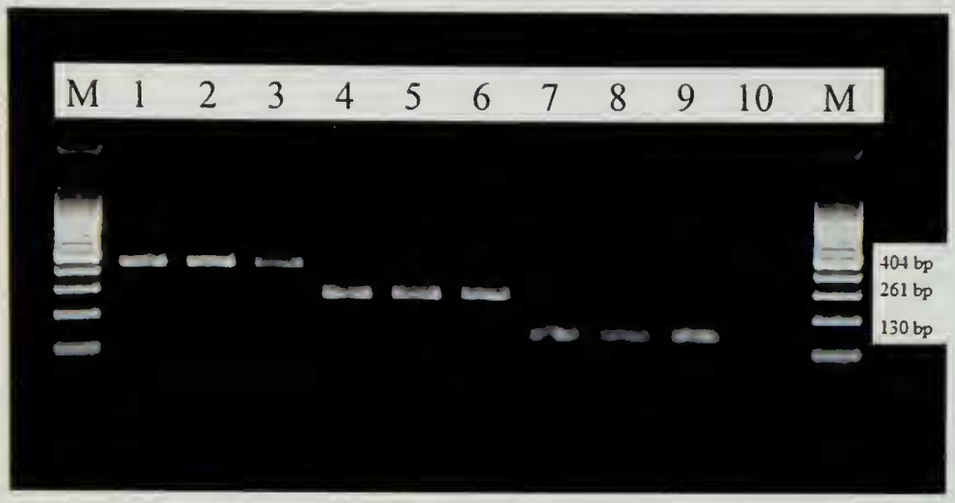

Figure 1. Specificity of multiplex PCR assay. Specific reverse primers for *A. ericaphidis*, *E. incompletus* and *P. unicum* were combined and tested with DNA from: *P. unicum*, Lanes 1–3; *E. incompletus*, Lanes 4–6; *A. ericaphidis*, Lanes 7–9. Lane 10 is a water control and Lanes M are 100-bp markers.

population of 13 *E. incompletus*, the average intraspecific divergence was 1.31% (range 0.3–2.4%), and seven haplotypes were identified: haplotype 1 (KC211027) was represented by six sequences, haplotype 2 (GU237131) was represented by two sequences, and haplotypes 3 (KC211028), 4 (KC211029), 5 (KC211030), 6 (KC 211031), and 7 (KC211032) were represented by one sequence each. Within the sample population of 37 *P. unicum*, the average intraspecific divergence was 0.42% (range 0.2–0.6%), and five haplotypes were identified: haplotype 1 (KC211020) was represented by 31 sequences, and haplotypes 2 (KC211021), 3 (KC211022), 4 (KC211023), and 5 (EU574904) were represented by 1, 1, 2, and 2 sequences, respectively.

The specific reverse primers designed for *A. ericaphidis*, *E. incompletus* and *P. unicum* (Table 1), when used individually with forward universal primer LCO, selectively amplified the DNA of the species for which they were designed. In the multiplex PCR assay, the combination of universal forward primer and all three specific reverse primers differentiated adults of *A. ericaphidis*, *E. incompletus* and *P. unicum*, and amplified the appropriate specific fragments: 130 bp for *A. ericaphidis*, 261 bp for *E. incompletus* and 404 bp for *P. unicum* (Fig. 1).

Validation of the multiplex PCR assay

Only three species of braconid primary parasitoids emerged from *E. fimbriata* collected from the Agassiz site (Fig. 2). Although other species of *Aphidius* and *Praon* have previously been reared from field-collected aphids in southwestern B.C. (Raworth *et al.* 2008), no additional species of these two genera were recovered in our study. As such, the primers we have developed can be used to identify the species of primary parasitoids from this site. *Praon unicum* emerged from the earliest collections of *E. fimbriata* in May, and was present in hosts throughout the collection period. *Aphidius ericaphidis* first emerged from hosts collected in late June, and was present throughout the rest of the collection period. A very small number of *E. incompletus* emerged from hosts collected in July, August and early September. A total of 10 hymenopteran parasitoid individuals (not shown in Fig. 2) that could not be identified by morphological characteristics emerged from aphids collected in May, June and early July. Secondary (hyper-) parasitoids (*Alloxysta* sp., as noted by Raworth *et al.* 2008) first emerged in late June, and were present every week (1–20 per week) until early September.

Peak numbers of one or both of the two dominant species, *A. ericaphidis* and *P. unicum*, emerged from hosts collected on 28 June, 20 July, 10 and 23 August, and 1 and 9 September (Fig. 2). To validate the multiplex PCR assay, DNA from 24–40 preserved *E. fimbriata* collected on each of 28 June, 20 July, 10 August and 1 September was challenged with the combined specific reverse primers (e.g., Fig. 3). On all four dates, multiplex PCR detection of percentage parasitism by either *A. ericaphidis* or *P.*

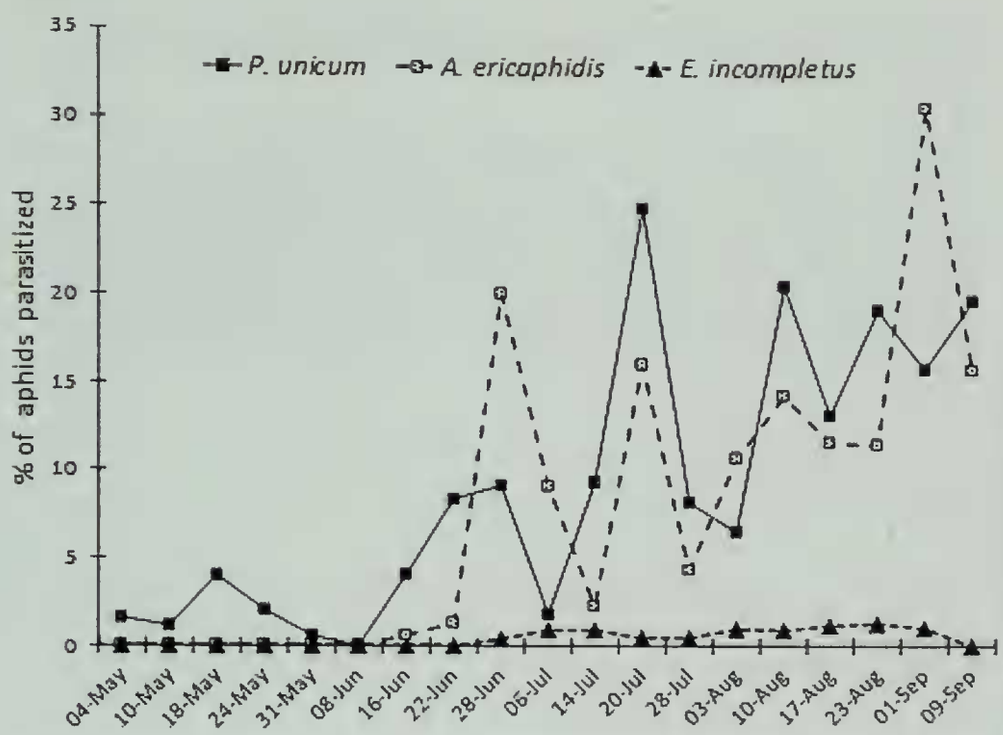

Figure 2. Percentage of field-collected *E. fimbriata* from which parasitoids emerged. Numbers of field-collected *E. fimbriata* reared from highbush blueberry were 62, 173, 50, 146, 174, 160, 174, 155, 250, 220, 225, 225, 232, 215, 225, 259, 157, 95 and 51, respectively, for each of the 19 weeks in 2011.

unicum was statistically similar to detection of parasitism by rearing field-collected *E. fimbriata* ($P>0.05$ in 8 individual comparisons; Table 2). The numbers of *E. incompletus* were too small for valid chi-square analysis (Fig. 2, Table 2). The multiplex PCR assay detected *E. incompletus* DNA on two of the four dates, whereas *E. incompletus* individuals were reared from aphids collected on each of the four dates. It is possible that primers in the multiplex assay did not detect some of the earliest, tiniest life stages (e.g., eggs) of *E. incompletus* or of the other two parasitoids. To establish detection limits of the primers, a third method of detection—dissection—should be compared with the multiplex PCR assay and the rearing methods (as per methods described by Gariepy *et al.* 2007 and Weathersbee *et al.* 2004). Additionally, sensitivity analysis of the multiplex PCR assay might help to explain some of the differences between the two detection techniques in our study.

The multiplex PCR assay detected DNA from both *A. ericaphidis* and *P. unicum* in a small percentage of *E. fimbriata* collected on 20 July, 10 August and 1 September (Table 2; Fig. 3, Lane 6), indicating multiparasitism by these two species. Stage-specific information about this competitive interaction between primary parasitoids could be obtained by dissecting the immature stages out of aphid hosts and challenging their DNA with the multiplex assay. More dynamic information about the outcomes of multiparasitism in field-collected *E. fimbriata* could be gathered by comparing a single-aphid rearing assay with dissection followed by the multiplex PCR assay. Rearing assays of multiparasitism by *A. smithi* and *P. pequodorum* on pea aphid, *Acyrthosiphon pisum*, showed that the survivor of competition between a first-instar *P. pequodorum* and any stage of *A. smithi* was *P. pequodorum*; but if *P. pequodorum* was killed in the egg stage, *A. smithi* survived (Chow and Mackauer 1984, 1985).

The trend toward increased multiparasitism between 20 July and 10 August suggests that fewer hosts were available because the *E. fimbriata* population was in decline due to seasonal changes in host-plant quality (Raworth 2004; Raworth and Schade 2006) and might indicate that parasitoid populations were increasing at that time.

Conclusions

The molecular diagnostics developed in this study can be used, in conjunction with rearing and dissection techniques, to conduct

Figure 3. Use of the multiplex PCR assay to detect parasitoid DNA in *E. fimbriata* collected on 20 July 2011 from highbush blueberry. Of 24 *E. fimbriata* analysed, 17 are represented in this figure. Results from one aphid are shown in each of Lanes 1–17. Lanes 1, 2, 5, 10, 11, 15, 16 and 17 show no parasitism (Lane 17 shows as a negative control: one *E. fimbriata* that was known to be unparasitized). Lanes 3, 7 and 13 show parasitism by *A. ericaphidis* (130 bp); Lane 9 shows parasitism by *E. incompletus* (261bp); Lanes 4, 8, 12 and 14 show parasitism by *P. unicum* (404 bp); and Lane 6 shows multiparasitism by *A. ericaphidis* and *P. unicum*. Lane 18 is a water control and Lanes M are markers. At the bottom of most lanes is a faint band (80 bp) from leftover PCR ingredients.

detailed studies of the population dynamics of the primary parasitoids of *E. fimbriata* in southwestern B.C., with or without the augmentative releases of *P. unicum* proposed by Raworth *et al.* (2008; and see Vafaie *et al.* 2013). In particular, it will be possible to track the immature stages of *A. ericaphidis*, *E. incompletus* and *P. unicum* in a single reaction using the multiplex PCR assay.

The diagnostic accuracy of the multiplex PCR could likely be improved by: sensitivity analysis of the PCR; analyses to determine if DNA from one species inhibits the PCR reaction to DNA of other species; and analysis of DNA from parasitoids congeneric to *A. ericaphidis*, *E. incompletus* and *P. unicum*.

Once these improvements have been made, the multiplex PCR would be useful on a larger geographical scale. *Aphidius ericaphidis* has been discovered in large numbers east and west of the Cascades, USA, as well as in southwestern B.C. (Raworth *et al.* 2008; Pike *et al.* 2011). *Praon unicum* has been reported from more than 30 different aphid hosts (see Smith 1944; Carroll and Hoyt 1986; Johnson 1987; Pike *et al.* 1997, 2000; Acheampong *et al.* 2012) besides *E. fimbriata*. The primers developed for *P. unicum* will, therefore, facilitate population studies in other systems.

Table 2

Number (%) of *E. fimbriata* from which a parasitoid emerged during rearing (R) compared to number (%) of *E. fimbriata* in which parasitoid DNA was detected by multiplex PCR assay (M).

Date (2011) of *E. fimbriata* collection	Species of parasitoid detected							
	P. unicum		*A. ericaphidis*		*E. incompletus*		*A.e.* plus *P.u.*[a]	
	R	M	R	M	R	M	R[b]	M
28 June	9.1	5.0	20.0	15.0	0.4	0	–	0
20 July	24.8	25.0	16.0	20.8	0.4	4.2	–	4.2
10 August	20.4	29.2	14.2	25.0	0.9	0	–	8.3
1 September	15.7	33.3	30.5	41.7	1.0	4.2	–	8.3

a Multiparasitism by *A. ericaphidis* and *P. unicum*.

b *Ericaphis fimbriata* was reared in groups, therefore the number of parasitoids emerging from one aphid could not be determined.

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